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# Prevalence, Implication, and Viral Nucleotide Sequence Analysis of GB Virus-C/Hepatitis G Virus Infection in Acute Fulminant and Nonfulminant Hepatitis

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The clinical impact of GB virus-C (GBV-C)/hepatitis G virus (HGV) infection on various causes of acute hepatitis and fulminant hepatitis is controversial. In this study, serum samples from 164 patients with acute hepatitis of various causes, 34 asymptomatic hepatitis B virus (HBV) carriers, and 34 healthy adults were tested for GBV-C/HGV RNA by reverse transcription-nested polymerase chain reaction using primers based on the 5′-untranslated region. Nucleotide sequences of GBV-C/HGV RNA from various groups were compared. The prevalence of GBV-C/HGV RNA was significantly higher in patients with acute hepatitis D virus (HDV) superinfection than in HBV carriers or healthy controls (10/37 vs. 2/34, $P < 0.02$; 10/37 vs. 1/34, $P < 0.005$). GBV-C/HGV RNA was detected in 11.1% of acute hepatitis A patients, 9.5% of acute hepatitis B patients, 15.8% of acute hepatitis C patients, 12.5% of acute hepatitis E patients, 11.8% of chronic hepatitis B patients with acute exacerbation, and 11.1% in patients with non-A to -E hepatitis; each was not significantly higher than that in HBV carriers or healthy adults. There were no significant differences in gender, age, serum albumin, bilirubin, and alanine aminotransferase levels nor in the occurrence of fulminant hepatitis (6/28 vs. 36/136) between patients with or without GBV-C/HGV RNA. All six patients with fulminant hepatitis who had GBV-C/HGV RNA were complicated by infection with hepatitis B, C, or D. The GBV-C/HGV clones from 21 patients with or without fulminant hepatitis belonged to group 3. No particular strain of GBV-C/HGV was associated with fulminant hepatitis. ***J. Med. Virol. 56:118–122, 1998.*** © 1998 Wiley-Liss, Inc.

**KEY WORDS:** flaviviridae, GBV-C/HGV, phylogenetic analysis, polymerase chain reaction, 5′-untranslated region, parenteral transmission, sexual transmission

## INTRODUCTION

GB virus-C (GBV-C)/hepatitis G virus (HGV) is related to the flaviviruses and is distantly related to hepatitis C virus (HCV) [Simons et al., 1995; Leary et al., 1996; Linnen et al., 1996] and can be grouped into the Flaviviridae family. Parenteral transmission is believed to be the main mode of spread of GBV-C/HGV because this virus is common in patients with a history of multiple blood transfusions or intravenous drug abuse [Simons et al., 1995; Linnen et al., 1996; Aikawa et al., 1996; Stark et al., 1996; Hwang et al., 1997; Kao et al., 1997]. Frequent sexual contact with multiple partners may be another important mode because GBV-C/HGV infection is also found frequently in prostitutes [Wu et al., 1997] and in non-drug-injecting homosexual and bisexual men [Stark et al., 1996]. Perinatal transmission of GBV-C/HGV from mother to their babies has also been reported [Feucht et al., 1996; Fischier et al., 1997].

GBV-C/HGV infection was found in 1% to 1.7% of blood donors and in 7% to 39% of patients with various types of acute and chronic hepatitis [Simons et al., 1995; Linnen et al., 1996; Aikawa et al., 1996; Fiordalisi et al., 1996; Kao et al., 1997]. However, the role of

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this virus in fulminant hepatitis remains controversial [Yoshiba et al., 1995; Kao et al., 1996a; Mishiro et al., 1996; Kuroki et al., 1996; Salle et al., 1996; Heringlake et al., 1996; Tameda et al., 1996; Kanda et al., 1997]. Many reports indicate that GBV-C/HGV infection does not modify the clinical course of coexisting HCV infection [Tanaka et al., 1996; Hwang et al., 1997; Kao et al., 1997; Francesconi et al., 1997; Goser et al., 1997; Saiz et al., 1997]. The prevalence and clinical impact of GBV-C/HGV infection in other types of acute hepatitis have not yet been investigated fully. In this study, serum GBV-C/HGV RNA was tested in patients with acute fulminant or nonfulminant hepatitis of various etiologies. The possible clinical impact of GBV-C/HGV infection on acute hepatitis was analyzed. Nucleotide sequences of GBV-C/HGV RNA from patients with acute fulminant or nonfulminant hepatitis were compared.

## MATERIALS AND METHODS

A total of 232 subjects (164 patients with acute hepatitis, 34 asymptomatic hepatitis B virus [HBV] carriers, and 34 healthy controls) were enrolled in this study (Table I). Patients with acute hepatitis were enrolled from inpatients admitted to Veterans General Hospital-Taipei. In the absence of a past history of hepatitis, these patients presented with symptomatic acute hepatitis and had elevated serum alanine aminotransferase (ALT) levels more than 10 times upper normal. The diagnosis of acute hepatitis A was based on the detection of immunoglobulin M antibody to hepatitis A virus (IgM anti-HAV). The diagnosis of acute hepatitis B was based on the detection of IgM antibody to hepatitis B core antigen (IgM anti-HBc) and the clearance of hepatitis B surface antigen (HBsAg) within 6 months of follow-up. The diagnosis of acute hepatitis C was based on seroconversion of antibody to hepatitis C virus (anti-HCV) or rising titer of anti-HCV. The diagnosis of acute hepatitis D virus (HDV) superinfection was based on the finding of seroconversion of antibody of HDV (anti-HDV) or rising titer of anti-HDV in chronic HBV carriers with acute hepatitis in the absence of IgM anti-HBc. The diagnosis of acute hepatitis E was based on the presence of antibody to hepatitis E virus (anti-HEV) in patients with acute hepatitis who were negative for IgM anti-HAV, HBsAg, IgM anti-HBc, anti-HCV, and anti-HDV. The diagnosis of chronic hepatitis B with acute exacerbation was based on the detection of HBV DNA in the absence of IgM anti-HBc, anti-HDV, and anti-HCV in chronic HBV carriers presenting with acute hepatitis. The diagnosis of non-A to -E hepatitis was based on the exclusion of all the above-mentioned causes of acute viral hepatitis, drug-induced liver injury, autoimmune hepatitis, and negative results for IgM antibodies to Epstein-Barr virus (EBV), cytomegalovirus, and herpes virus. Asymptomatic HBV carriers were subjects with persistence of HBsAg and normal ALT levels for more than 6 months. Healthy adults did not have serological markers of current hepatitis viral infection and their ALT levels were normal. The latter two groups visited this hospital for routine check-up.

Serum GBV-C/HGV RNA was detected by nested polymerase chain reaction following reverse transcription (RT-PCR). Outer (primer 3F 5′-GGCCAAAAG-GTGGTGGATGG, primer 4R 5′-GAGCTGGGTGGC-CCCATGCA) and inner primers (primer S2 5′-GGTTGGTAGGTCGTAAATCC, primer A2 5′-CGTACGTGGGCGTCGTTTGC) based on the 5′-untranslated region were used [Muerhoff et al., 1996]. The RT-PCR products were analyzed in 3% agarose gel, followed by ethidium bromide staining. The estimated size of the RT-PCR products of GBV-C/HGV RNA was 203 base pair. Strict procedures were followed to avoid false positive results [Kwok and Higuchi, 1989]. The amplified PCR products were ligated into the plasmid pCR2 vector (Original TA Cloning® Kit, Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. The ligation mixture was used to transform the competent *Escherichia coli* strain DH5 α (Gibco BRL, Life Technologies, Gaithersburg, MD) [Chung and Miller, 1988; Wu et al., 1995]. One or more positive colonies were picked up and cultured in Lauria-Bartani medium. Plasmid DNA was extracted and subjected to the dye terminator cycle sequencing reaction according to the standard protocol provided by the manufacturer (Dye terminator cycle sequencing core kit no. 402117, Perkin Elmer Cetus Corp., Norwalk, CT). The sequencing products were precipitated with alcohol and analyzed by an ABI 373A sequencer (Perkin Elmer). GBV-C/HGV sequences were aligned by multiple alignments using the Clustal V Program [Higgins et al., 1992]. The neighbor-joining method using the Molecular Evolutionary Genetics Analysis Program [MEGA, Version 1.01, Kumar et al., 1993] was applied for phylogenetic analysis of sequencing data. The confidence of the clades, i.e., the monophyly, was tested by bootstrapping (a resampling statistical technique) with 1,000 replicates of heuristic searches. The nodes with bootstrap values greater than 70% are significantly supported with ≧95% confidence (robustness) [Hillis and Bull, 1993].

The following viral markers were tested by radioimmunoassay kits: IgM anti-HAV, HBsAg, IgM anti-HBc, and anti-HDV (HAVABM, Ausria II-125, CORAB-M, and anti-Delta; Abbott Laboratories, North Chicago, IL). Anti-HCV was examined by a second-generation enzyme immunoassay (Abbott Laboratories). Anti-HEV was measured by a solid-phase enzyme-linked immunosorbent assay (ELISA) (Genelabs and Diagnostic Technology, Singapore). IgM antibody to EBV (EBV IgM; Gull Laboratories, Salt Lake City, UT) was carried out by immunofluorescence. IgM antibodies to cytomegalovirus and herpes virus were carried out by sandwich enzyme immunoassays (cytomegalovirus-IgM-ELISA, HSV IgM; Human Gesellschaft for Biochemical and Diagnostical mbH, Taunusstein, Germany). Antinuclear antibody, anti-smooth muscle antibody, and anti-mitochondria antibody were carried out by an indirect fluorescent antibody technique

(FLUORO-KIT™, Incstar Corporation, Stillwater, MN). Serum ALT, albumin, and bilirubin levels were measured by a sequential multiautoanalyzer (Technicon SMAC; Technicon Instruments, Tarrytown, NY).

## RESULTS

Serum samples from 164 patients with acute hepatitis, 34 asymptomatic HBV carriers, and 34 healthy adults were tested for the presence of GBV-C/HGV RNA by a nested RT-PCR using primers based on the 5′-untranslated region. A total of 28 (12.1%) patients had detectable viremia. The prevalence of GBV-C/HGV infection in each group ranged from 2.9% in healthy adults to 27% in patients with acute HDV superinfection (Table I). It was significantly higher in patients with acute HDV superinfection than in HBV carriers ($P < 0.02$) or healthy controls ($P < 0.005$). There were no significant differences in the detection rates of GBV-C/HGV RNA among the remaining groups with acute hepatitis, HBV carriers, and healthy controls. Of the 27 patients with non-A to -E hepatitis, 3 (11.1%) had GBV-C/HGV infection. Although these three patients were negative for both HBsAg and anti-HCV, one was positive for serum HCV RNA and the second was positive for HBV DNA by PCR. Potential risk factors associated with GBV-C/HGV infection within 6 months before acute hepatitis infection were assessed in the 25 hepatitis patients who had detectable GBV-C/HGV RNA. Eleven patients had a history of parenteral exposure (operation, injection, tattooing, acupuncture, dental procedures, or blood transfusion) and six (all with HDV superinfection) had a history of sexual contact with prostitutes within 6 months of acute hepatitis infection. The risk factors associated with GBV-C/HGV infection in the remaining eight patients with hepatitis who were also positive for GBV-C/HGV RNA were unclear.

The biochemical data and clinical course of patients with acute hepatitis with or without GBV-C/HGV RNA are shown in Table II. There were no significant differences in gender, age, serum albumin, bilirubin, and ALT levels between patients with or without GBV-C/HGV RNA. Only 6 (24%) of the patients with GBV-C/HGV RNA presented with fulminant hepatitis, while 36 (25.9%) of the patients without GBV-C/HGV RNA presented with fulminant hepatitis. Of the six patients with fulminant hepatitis who had GBV-C/HGV RNA, three had coexisting HDV superinfection, two had chronic hepatitis B with acute exacerbation, and one had acute posttransfusion hepatitis C.

GBV-C/HGV RNA genomes from 5 patients with fulminant hepatitis, 14 patients with acute nonfulminant hepatitis, an asymptomatic HBV carrier, and a healthy control were cloned and sequenced. All the GBV-C/HGV clones isolated from our laboratory were named with Taiwan (TW) followed by a serum sample number. TW406G and TW1460G were serum samples taken 26 months apart from the same patient with fulminant hepatitis caused by acute HDV superinfection. The remaining serum samples were from 20 different, irrelevant subjects. Phylogenetic analysis using MEGA was conducted on these isolates and isolates obtained from GenBank (Fig. 1). There are three major monophyletic groups supported significantly by bootstrap values greater than 70%. All the GBV-C/HGV isolates from the patients with or without fulminant hepatitis and the isolates from Japan belonged to group 3 [Orito et al., 1996; Smith et al., 1997; Muerhoff et al., 1997]. The isolates from the United States and Europe formed group 2. The isolates from West Africa constituted group 1. No particular strain of GBV-C/HGV was associated with fulminant hepatitis.

TABLE I. Prevalence of GBV-C/HGV Infection in Patients With Acute Hepatitis and in Controls†

| Groups | No. of cases | No. (%) with GBV-C/HGV RNA |
|---|---|---|
| Acute hepatitis A | 18 | 2 (11.1) |
| Acute hepatitis B | 21 | 2 (9.5) |
| Acute hepatitis C | 19 | 3 (15.8) |
| Acute hepatitis D | 37 | 10 (27.0)* |
| Acute hepatitis E | 8 | 1 (12.5) |
| CHB with exacerbation | 34 | 4 (11.8) |
| Non-A to non-E hepatitis | 27 | 3 (11.1) |
| Hepatitis B carriers | 34 | 2 (5.9)** |
| Healthy adults | 34 | 1 (2.9)*** |

†CHB, chronic hepatitis B; acute hepatitis D, chronic hepatitis B with acute HDV superinfection.
* vs. **, $P < 0.02$; * vs. ***, $P < 0.005$.

TABLE II. Comparison of Clinical Profile and Biochemical Data of Acute Hepatitis Patients With or Without GBV-C/HGV RNA*

| Clinical data | With | Without |
|---|---|---|
| No. of cases | 25 | 139 |
| No. with male gender | 18 (72.0%) | 97 (69.8%) |
| Age (mean ± SD, years) | 42.3 ± 19.4 | 45.5 ± 17.1 |
| Albumin (mean ± SD, g/dl) | 3.6 ± 0.7 | 3.5 ± 0.7 |
| ALT (mean ± SD, U/L) | 2,158 ± 2,317 | 1,947 ± 1,693 |
| Bilirubin (mean ± SD, mg/dl) | 13.3 ± 12.3 | 14.3 ± 13.5 |
| Fulminant hepatitis | 6 (24.0%) | 36 (25.9) |

*No significant difference was seen in gender, age, albumin, ALT, bilirubin, or fulminant hepatitis between patients with or without GBV-C/HGV RNA.

## DISCUSSION

In this study, the detection of GBV-C RNA/HGV RNA was carried out by RT-PCR using primers based on the 5′-untranslated region, which was reported to give the highest sensitivity [Muerhoff et al., 1996, 1997; Smith et al., 1997]. The prevalence of GBV-C/HGV RNA in healthy adults and asymptomatic HBV carriers in this study was 2.9% and 5.9%, respectively, similar to previous reports from this area [Hwang et al., 1997; Kao et al., 1997; Wu et al., 1997] and elsewhere [Simons et al., 1995; Linnen et al., 1996; Aikawa et al., 1996; Fiordalisi et al., 1996]. There were no significant differences in the prevalence of GBV-C/HGV RNA in two control groups and in patients with acute hepatitis except acute HDV superinfection. This is the first study to find a high prevalence (27%) of GBV-C/

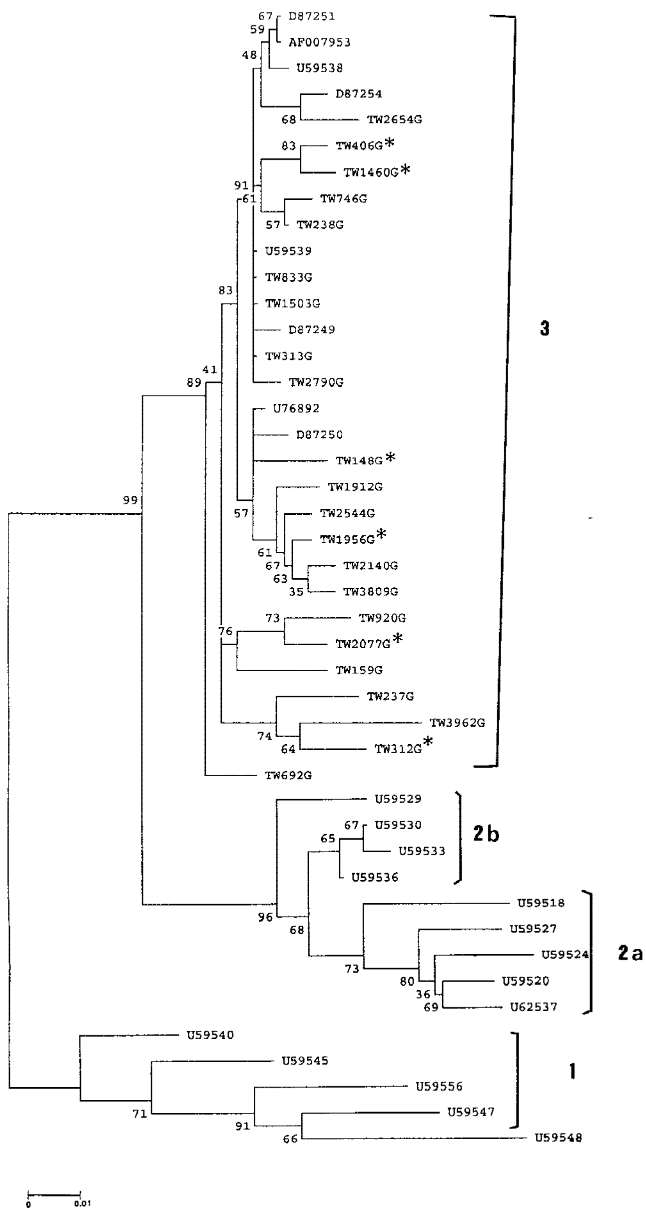


Fig. 1. Phylogenetic analysis of the GBV-C/HGV isolates from Taiwan and other geographic areas in the world. The neighbor-joining method using MEGA Version 1.01 [Kumar et al., 1993] was applied for phylogenetic analysis of sequencing data [Swofford et al., 1996]. The confidence of the clades, i.e., the monophyly, was tested by bootstrapping with 1,000 replicates of heuristic searches. The number on each node indicates a bootstrap value (shown by percentage). The nodes with bootstrap values greater than 70% are significantly supported with ≧95% confidence [Hillis and Bull, 1993]. TW, Taiwan. The numbers other than Taiwanese isolates indicate GenBank number of GBV-C/HGV isolates. Asterisk indicates isolates from patients with fulminant hepatitis. TW406G and TW1460G were serum samples taken 26 months apart from the same patient with fulminant hepatitis caused by acute HDV superinfection. The remaining serum samples were taken from 20 different, irrelevant subjects.

HGV RNA in patients with acute HDV superinfection who had a history of sexual contact with prostitutes. It has been reported that GBV-C/HGV RNA is also prevalent (21%) in prostitutes [Wu et al., 1997] and that sexual contact with prostitutes is the most common transmission route for HDV infection in this area [Wu et al., 1990]. GBV-C/HGV may be transmitted with HDV through sexual contact with prostitutes. Although risk factor analysis indeed revealed parenteral exposure as the main cause for GBV-C/HGV infection, sexual contact appeared to be another transmission route in our patients.

Our findings of no significant differences in biochemical data and the occurrence of fulminant hepatitis between hepatitis patients with or without GBV-C/HGV RNA support the belief that GBV-C/HGV infection does not modify the clinical course of coexisting HCV infection [Tanaka et al., 1996; Hwang et al., 1997; Kao et al., 1997; Francesconi et al., 1997; Goser et al., 1997; Saiz et al., 1997]. Our findings also indicate that GBV-C/HGV infection does not influence significantly clinical manifestations of acute hepatitis caused by different viruses and was not related significantly to fulminant hepatitis [Kao et al., 1996a; Mishiro et al., 1996; Kuroki et al., 1996; Salle et al., 1996; Kanada et al., 1997].

Kao et al. [1996b] reported phylogenetic analysis of GBV-C/HGV based on partial sequences of the NS3 region. They concluded that 81% of the Taiwanese isolates were more closely related to the East African isolate and could be classified into at least three groups. According to recent reports [Orito et al., 1996; Smith et al., 1997; Muerhoff et al., 1997], such analysis should be based on the 5′-untranslated region, since partial sequences of the coding region may not be reliable for the reconstruction of phylogenetic relationships of GBV-C/HGV. In this study, phylogenetic analysis of GBV-C/HGV clones from Taiwan and other geographic areas was conducted based on 5′-untranslated region and resulted into 3 major monopyletic groups significantly supported by bootstrap values, consistent with previous reports [Orito et al., 1996; Smith et al., 1997; Muerhoff et al., 1997]. However, all viral clones from our patients with or without fulminant hepatitis were close to the isolates from Japan and belonged to group 3 GBV-C/HGV. The East African isolate and some isolates from the United States and Europe belonged to group 2b. Both genotypes I and II HDV in Taiwan were also found to share the most common ancestor with the Japanese strains [Wu et al., 1998]. The close relationships between Taiwanese and Japanese strains of GBV-C/HGV and HDV can be explained historically and geographically. More GBV-C/HGV isolates from Taiwan need to be analyzed to determine if there are strains other than group 3.

An association between infection with some particular strains of GBV-C/HGV and the occurrence of fulminant hepatitis has been reported [Yoshiba et al., 1995; Heringlake et al., 1996; Tameda et al., 1996]. In this study, phylogenetic analysis gave no evidence of the association of fulminant hepatitis with any particular strain of GBV-C/HGV. Moreover, all six patients with fulminant hepatitis and GBV-C/HGV RNA were complicated by hepatitis B, C, or D infection. GBV-C/HGV RNA did not seem to be the cause of fulminant hepatitis. These findings argue strongly against GBV-C/

HGV being related to fulminant hepatitis either by itself or in concert with other known hepatitis viruses. Interestingly, two of the three patients with the diagnosis of non-A to -E hepatitis who had GBV-C/HGV RNA also had detectable HBV DNA or HCV RNA by PCR or RT-PCR. Acute hepatitis in these two patients was caused more likely by HBV and HCV than by GBV-C/HGV. The rare association of GBV-C/HGV with hepatitis may actually be due to other known or unknown coexisting hepatitis viruses if PCR assays are used besides conventional serological markers.

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